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[D-Ala^2]-Methionine Enkephalinamide (DALA): Characterization of Antinociceptive, Cardiovascular, and Autonomic Nervous System Actions in Conscious and Pentobarbital-Anesthetized Rats

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RANDICH, A. AND M. F. CALLAHAN. *[D-Ala^2]-Methionine enkephalinamide (DALA): Characterization of antinociceptive, cardiovascular, and autonomic nervous system actions in conscious and pentobarbital-anesthetized rats.* PHARMACOL BIOCHEM BEHAV **25**(3) 641–650, 1986.—The antinociceptive, cardiovascular, and autonomic nervous system actions of [D-Ala^2]-methionine enkephalinamide (DALA) were assessed in conscious and pentobarbital-anesthetized rats. Intravenous administration of DALA inhibited the tail-flick reflex evoked by noxious radiant heat in both conscious and pentobarbital-anesthetized rats. In general, the magnitude of the inhibition was not significantly affected by the presence of pentobarbital-anesthesia. DALA also induced hypotension and bradycardia in these rats, but these responses were significantly attenuated by pentobarbital-anesthesia. Arterial blood flows were initially decreased and vascular resistances increased in mesenteric, renal, and hindquarter beds following DALA administration, but these parameters rapidly returned to baseline levels except in the hindquarter bed where flows increased significantly above baseline levels. All of the changes in blood flows were significantly greater in conscious compared to pentobarbital-anesthetized rats. In intact pentobarbital-anesthetized rats, DALA induced inhibition of renal sympathetic nerve activity. However, DALA induced a pressor response with a brief increase in renal sympathetic nerve activity in rats with bilateral vagotomy. Similarly, in the conscious rat with ganglionic blockade, DALA induces a brief pressor response. These outcomes indicate that the brief hypotension observed in the intact rat following administration of DALA is probably the net outcome of a large vagally-induced decrease in sympathetic tone and a small increase in sympathetic tone of either neurogenic or peripheral origin. These outcomes are discussed in terms of cardiovascular-somatosensory interactions.

Antinociception DALA Vagus Regional blood flow Renal sympathetic nerve Anesthesia

INTRAVENOUS (IV) administration of [D-Ala^2]-methionine enkephalinamide (DALA: 22 or DAME: 14) results in inhibition of the tail-flick reflex evoked by noxious radiant heat, reflex bradycardia, and hypotension in conscious rats [23]. Similar cardiovascular responses are obtained following IV DALA administration in either urethane-anesthetized or decerebrate rats [33,34]. The cardiovascular responses evoked by DALA may be due to activation of pulmonary J receptors [20], since resection of the vagi below the cardiac branches eliminates the cardiovascular actions of DALA in an open-chest preparation [34]. The hypotensive effect of DALA also occurs in urethane-anesthetized, atropinized, paralyzed, and artificially ventilated rats suggesting that the hypotension is not a secondary consequence of opioid-induced reflex bradycardia [33]. This view is also supported by recordings of activity of the greater splanchnic nerve, which decreased following administration of DALA and was mediated by activation of vagal afferents. Finally, both the cardiovascular and antinociceptive actions of DALA can be blocked by either prior administration of the opiate receptor antagonist naloxone or bilateral cervical resection of the vagi, thereby suggesting a specific action of DALA on opioid receptors perhaps located within the cardiopulmonary region.

Central administration of DALA also can evoke antinociceptive and cardiovascular responses. Microinjection of DALA in the periaqueductal gray region of conscious rats results in antinociception as indexed by inhibition of the tail-flick reflex evoked by noxious radiant heat, increases in jump-squeal thresholds evoked by electric shock, and increased vocalization thresholds evoked by hind-limb pinch [22]. Intracerebroventricular (ICV) administration of DALA

in urethane-anesthetized rats results in the cardiovascular responses of bradycardia and a triphasic arterial blood pressure response of hypotension-hypertension-hypotension [4]. These peripheral and central actions of DALA are consistent with the more general view that activation of cardiopulmonary afferents produces both circulatory adjustments and modulation of somatosensory input, thereby forming a potential substrate for adaptive responses to physical and psychological stressors (see [24,25] for reviews).

The purpose of the following experiments was to further characterize antinociceptive, cardiovascular, and autonomic nervous system actions of DALA administered IV in conscious and pentobarbital-anesthetized rats. The vagally-mediated antinociceptive actions of IV DALA are of particular interest in pain research because enkephalins and enkephalin-like substances exist in peripheral tissue, and can be released either *in situ* or into the circulation following either physiological or pharmacological activation of sympathetic nerves [6, 9, 10]. Since these substances do not readily cross the blood-brain barrier and are rapidly degraded [18, 21, 27], the vagal afferent mechanism described for DALA antinociception provides a potential means by which endogenous enkephalins and enkephalin-like substances could exert antinociceptive actions. Moreover, since the antinociceptive action of DALA is probably mediated by vagal input to central nervous systems (CNS) of descending inhibition (see [7,24] for reviews), the analysis of CNS substrates responsible for the antinociception will most likely involve neural tissues that can be manipulated only in the anesthetized rat. For example, rats do not regain consciousness following bilateral lesions of the caudal lateral reticular nucleus (LRN) and this is an established medullary substrate of both pain inhibition [8,29] and cardiovascular control [28]. Therefore, one specific purpose of these studies was to compare inhibition of the tail-flick reflex evoked by noxious radiant heat in conscious and pentobarbital-anesthetized rats following IV DALA administration to ensure comparability of these two preparations and hence, trans-preparation generalization of outcomes (see [29,30] for a discussion of the importance of the pentobarbital-anesthetized preparation in pain research and a similar analysis of stimulation-produced analgesia).

Similarly, the vagally-induced cardiovascular actions of DALA administered IV are of particular interest in cardiovascular research. Enkephalins and enkephalin-like substances administered to intact and decerebrate rats and cats exert multiple effects depending upon the substance, route, and site of administration. The responses range from tachycardia and hypertension to bradycardia and hypotension [4, 12, 19, 23, 31, 33, 34]. These substances and responses have been demonstrated to be critical components of such cardiovascular phenomena as hemorrhagic and endotoxic shock (for review [14]). Therefore, comparisons were also made of heart rate, arterial blood pressure, and peripheral arterial blood flows (mesenteric, renal, and hindquarter vascular beds) in both conscious and pentobarbital-anesthetized rats. These data were also used to calculate arbitrary changes in resistance of the various beds, thereby providing further characterization of the cardiovascular actions of DALA and data that bear on the issue of how changes in blood flow might influence the tail-flick reflex independent of any antinociceptive action of DALA. Finally, renal sympathetic nerve recordings were made in both intact and bilaterally vagotomized pentobarbital-anesthetized rats to more fully characterize and quantify the autonomic nervous system actions of DALA.

METHOD

Subjects

Experimentally-naive male Sprague-Dawley rats obtained from Hormone Assay Laboratories in Chicago served as subjects. The rats were individually housed in wire-mesh cages under a 12:12 hr light-dark cycle. Food and water were available on an ad lib basis.

Apparatus

Nociceptive responses were measured with a tail-flick apparatus. The radiant heat stimulus was provided by a 500 W projector bulb housed in a metal casing and focused on the rats' tail through a small opening in the metal housing. Onset and termination of each trial were controlled automatically by a digital timer. The intensity of the radiant heat stimulus, which was constant for all subjects, was intended to produce a tail-flick response of approximately 4 sec in a conscious rat.

Arterial blood pressure and heart rate were recorded on a Beckman R11A rectilinear dynagraph from the pulse pressure signal provided by a Century pressure transducer.

Arterial blood flows were obtained with minaturized pulsed Doppler flow probes (Valpey Fisher, Hopkinton, MA) and a pulsed Doppler flowmeter (University of Iowa, Iowa Biomedical Engineering, University of Iowa, Iowa City, IA) which measures blood cell velocity as a change in frequency (e.g., doppler shift) that is directly proportional to the blood flow in the vessel.

Multifiber nerve activity was amplified with a differential preamplifier (Data Inc.) with the lower and upper bandpass filters set at 30 and 10,000 Hz, respectively. The signal from the preamplifier was visualized on a storage oscilloscope and passed to a nerve traffic analyzer equipped with a window discriminator and a microprocessor based counter and frequency meter. Multifiber sympathetic activity was quantified as spikes/sec, employing 1-sec time bins.

Surgical Techniques

Each rat was anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg). Catheters were implanted into the left common carotid artery (Microline) and the external jugular vein (Silastic). Details of this procedure are reported elsewhere [23]. The arterial and venous cannulae were then drawn subcutaneously around the neck and exited through a dorsal incision. The cannulae were anchored to the neck and flushed with a saline-heparin solution.

In studies involving measurements of blood flow, rats received a laparotomy followed by implantation of minaturized pulsed doppler flow probes on the superior mesenteric artery, the left renal artery, and the lower abdominal aorta to determine blood flow in these individual vascular beds. The blood flow in the lower abdominal aorta is used as a measure of blood flow to skeletal muscle in the hindquarters, as this is the major bed serviced by the vessel [5, 11, 13]. In the conscious preparation, arterial and venous catheters were implanted 5 to 10 days after insertion of the pulsed doppler flow probes (after the rats had attained at least 90% of preoperative body weight).

In studies of sympathetic renal nerve recording, rats received a laparotomy, and the viscera were retracted to expose the aorta and vena cava at the renal bifurcations. The tissue immediately rostrad to the renal artery and lateral to the celiac ganglion was gently retracted to expose underlying

renal sympathetic nerves. A nerve was gently placed over a platinum-irridium hook electrode and activity was recorded. The electrode assembly was then covered with polysiloxane vinyl (Reprosil) to ensure stability of the preparation.

Testing

Experiment 1. Experiment 1 compared the antinociceptive and cardiovascular responses (heart rate and arterial blood pressure) produced by IV administration of 500 μg/kg of DALA in conscious and pentobarbital-anesthetized rats. Dose response functions for DALA have been reported previously for these measures in the conscious rat [23] and a 500 μg/kg dose was chosen as the stimulus for the present experiments because it produces robust antinociception. Rats in the conscious condition were tested 24 hr after recovery from both catheter implantation and a sham laparotomy (see ahead). Each rat was placed in a Plexiglas restraining tube and the cannulae were connected. Rats in the pentobarbital-anesthetized condition were tested approximately 40–50 min following injection of 50 mg/kg of pentobarbital sodium and implantation of arterial and venous cannulae, and flow probes. These animals were not restrained, but all other procedures were identical to those described for rats in the conscious condition. Arterial blood pressure and heart rate were continuously recorded. Tail-flick trials were then administered until baseline latencies were approximately 4 sec and remained stable from trial to trial. Following stabilization of the tail-flick response, each rat received successive bolus IV injections of isotonic saline and 500 μg/kg of DALA (Sigma Co.). DALA dissolved in isotonic and saline vehicle were infused in a volume of 100 μl/kg body weight at a rate of approximately 100 μl/sec. Each drug or saline injection was followed by a 150–200 μl saline flush. Tail-flick trials were administered 0.25-, 1-, 2-, 3-, 4-, and 5-min after saline or drug. A maximum tail-flick latency of 10 sec was used to prevent tissue damage to the tail (approximately 2.5 × threshold).

Experiment 2. Experiment 2 compared arterial blood flows in the superior mesenteric artery, the left renal artery, and the abdominal aorta following IV administration of DALA in conscious and pentobarbital-anesthetized rats. Rats in the conscious condition were tested 48 hr after catheter implantation and 5–10 days after flow probe implantation as noted previously. Each rat in the conscious condition was tested in its home cage while quiet. The testing procedure was identical to that described previously in Experiment 1, except that only cardiovascular measures were taken at the 0.25-, 1-, 2-, 3-, 4-, and 5-min time points. The identical procedure was used for rats in the pentobarbital-anesthetized condition, except that the measures were taken approximately 40–50 min after the original anesthesia injection and at a time when tail-flicks trials were being administered in Experiment 1. All cardiovascular measures were taken precisely at the start of a trial to eliminate any possible influence of the heat stimulus on the responses.

Experiment 3. Experiment 3 assessed renal sympathetic nerve activity following either isotonic saline or IV DALA administration in both sham vagotomized and bilaterally vagotomized rats under light pentobarbital anesthesia. Each rat in the sham condition was tested after dissipation of the anesthesia to the approximate level used in the previous experiments (lid reflexes present). Renal nerve activity, heart rate and blood pressure were recorded at 0.25-, 1-, 2-, 3-, 4-, and 5-min after the saline or drug dose. Rats in the bilateral vagotomy condition were treated similarly, except that bilateral resection of the cervical vagi was performed approximately 15 min prior to any recording. In addition, rats in the sham vagotomy condition received a bolus injection of 31.25 μg/kg of phenylephrine to provide comparison data for the DALA treatment. Following the experiment, each rat was administered 20 mg/kg of hexamethonium bromide to insure that recordings were from post-ganglionic fibers. Some of the rats also received an additional administration of 500 μg/kg of DALA to assess whether arterial blood pressure responses were still manifested in the presence of ganglionic blockade. The rats were then sacrificed by an overdose of pentobarbital and nerve activity was recorded for an addi-

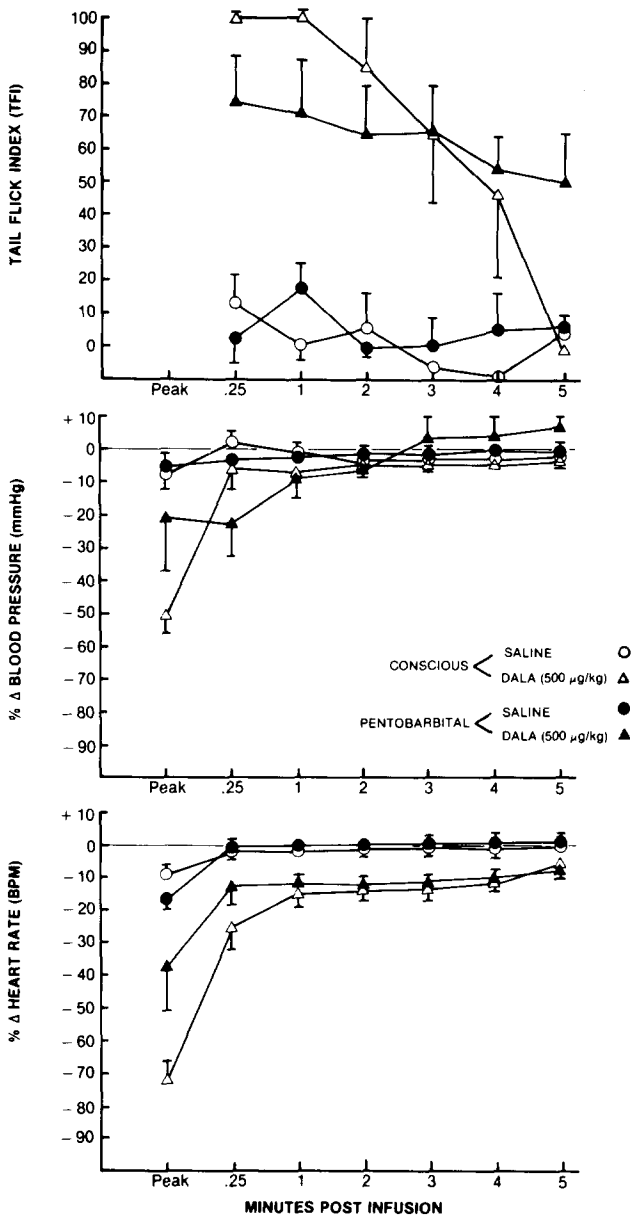

FIG. 1. Mean tail-flick index (top panel), mean percent change in arterial blood pressure (middle panel), and mean percent change in heart rate (bottom panel) as function of either saline or DALA (500 μg/kg) administration in conscious and pentobarbital-anesthetized rats.

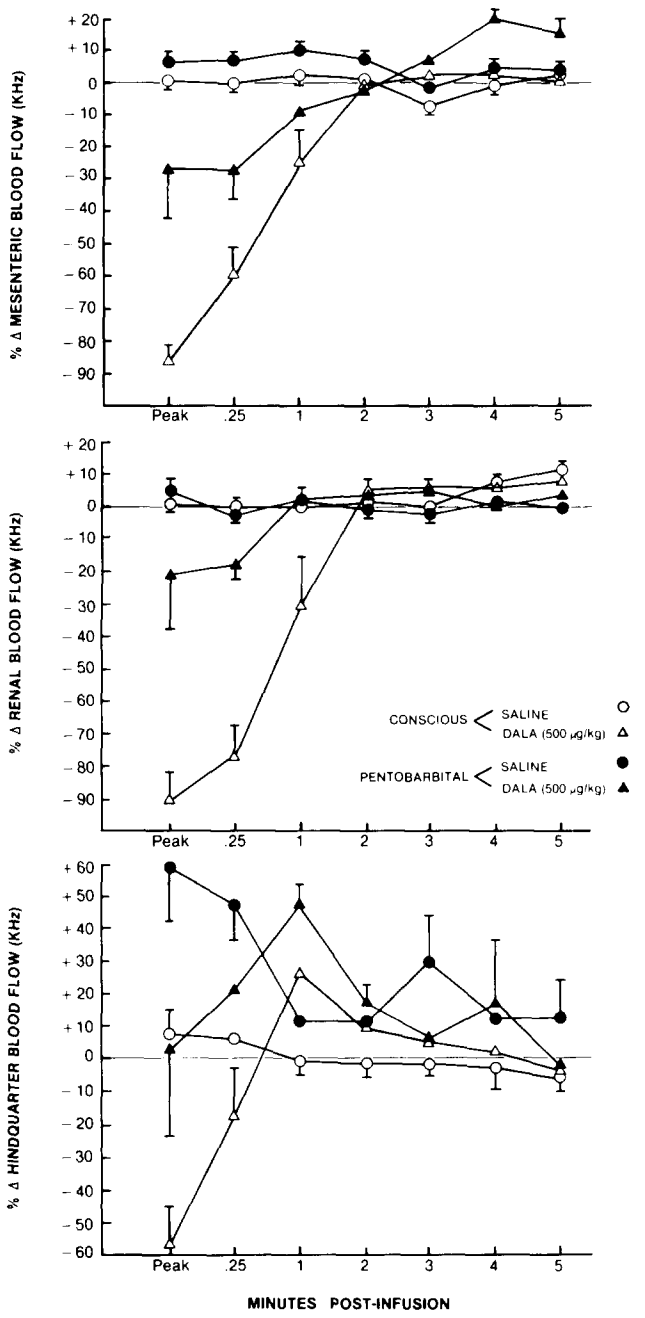

FIG. 2. Mean percent change in arterial blood flows (kHz of Doppler shift) in the mesenteric (top panel), renal (middle panel), and hindquarter (bottom panel) vascular beds as a function of either saline or DALA (500 μg/kg) administration in conscious and pentobarbital-anesthetized rats.

tional 30 min. Any recorded activity at the end of this period was subtracted from experimental data and considered to be noise.

Data Analysis

Tail-flick latencies were converted to tail-flick indices by the equation ((trial latency − baseline latency)/(10 seconds − baseline latency) × 100)). In addition to the cardiovascular measures obtained at the 0.25-, 1-, 2-, 3-, 4-, and 5-min trial points, the largest cardiovascular responses (heart rate, blood pressure, blood flows, resistance to conductance) and sympathetic responses manifested prior to the 0.25-min trial point were recorded and defined as the "Peak" responses.

Vascular resistance in each vascular bed was called resistance to conductance and calculated according to the following formula: resistance to conductance (arbitrary units) = MAP/kHz Doppler shift [13]. Resistance to conductance was used because in the present circumstances large decreases in heart rate may have contributed to the outcomes, i.e., these data may not reflect pure resistance changes. Tail-flick indices, percentage change from baseline blood pressure, percentage change from baseline heart rate, percentage change from baseline flows, percentage change from baseline resistance, and percentage change from baseline renal nerve activity (where peak responses are also included for cardiovascular and sympathetic nerve measures) were subjected to analysis of variance. In each analysis in which the overall null hypothesis was rejected, Scheffe post-hoc comparisons were performed on the means. Alpha was set at 0.05.

RESULTS

Figure 1 presents the outcomes of the first experiment comparing the tail-flick, arterial blood pressure and heart rate responses of conscious and pentobarbital-anesthetized rats following administration of either saline vehicle or DALA (500 μg/kg).

The top panel presents mean tail-flick indices evoked by either saline or drug at the six test trial time points. Group mean baseline tail-flick latencies obtained immediately prior to the drug trials were: conscious-saline=4.20; conscious-DALA=4.34; pentobarbital-saline=4.37; and pentobarbital-DALA=4.12, sec. An ANOVA indicated no significant baseline differences as a function of either anesthesia (conscious versus pentobarbital), $F(1,8)=0.01$, or drug (saline versus DALA), $F(1,8)=0.10$. The top panel of Fig. 1 shows that DALA produced inhibition of the tail-flick reflex in both conscious and pentobarbital-anesthetized rats. Saline administration produced little effect on this response. These views were confirmed by a mixed design ANOVA using factors of anesthesia (conscious versus pentobarbital), drug (saline versus DALA) and time (0.25-, 1-, 2-, 3-, 4-, and 5-min). This analysis revealed significant effects of drug, $F(1,8)=97.65$; time, $F(5,40)=6.90$; anesthesia × time, $F(5,40)=2.66$; and drug × time, $F(5,40)=4.73$. Since the interaction terms were significant, one way ANOVAs were carried out at each test trial time. Significant ANOVAs were obtained at all trials, and were then followed by mutually orthogonal Scheffe post-hoc analyses of means. These analyses revealed the following pattern of outcomes: on test trial times 0.25-, 1-, 2-, 3-, and 4-min, the two saline means (conscious and pentobarbital) did not significantly differ (Fs ranged from 0.00 to 0.54); the means of the two DALA treatments did not differ (Fs ranged from 0.00 to 1.61); and the two saline means combined differed significantly from the means of the two DALA treatments combined (Fs ranged from 3.34 to 29.23). On test trial time 5-min, the means of the two saline treatments did not differ ($F=0.00$); the means of the two saline treatments combined did not differ from the mean of the conscious-DALA treatment ($F=0.13$); but these latter three treatment means combined differed significantly from the pentobarbital-DALA mean ($F=5.06$). In summary,

there are no significant differences between the TFI values of pentobarbital-treated and conscious rats administered DALA except at the 5-min test trial, when the pentobarbital-anesthetized rats still manifest significant antinociception compared to both controls and conscious rats treated with DALA.

The middle panel of Fig. 1 presents mean arterial blood pressure responses evoked by saline and DALA expressed as percentage change from baseline values. In general, DALA induced a short-lived hypotension in both conscious and pentobarbital-anesthetized rats. Mean baseline arterial blood pressures obtained immediately prior to the drug trials were: conscious-saline=120; conscious-DALA=121; pentobarbital-saline=129; and pentobarbital-DALA=126, mmHg. Thus, anesthesia had no obvious effect on baseline arterial blood pressures and this view was confirmed by an ANOVA on baseline arterial blood pressures that indicated no significant differences as a function of level of anesthesia, $F(1,8)=0.39$, or drug, $F(1,8)=6.00$. A mixed design ANOVA of percentage change measures obtained following administration of the drugs indicated a significant effect of time, $F(6,48)=19.52$; a significant anesthesia × time interaction, $F(6,48)=5.59$; a significant drug × time interaction, $F(6,48)=18.39$; and a significant anesthesia × drug × time interaction, $F(6,48)=4.27$. One way ANOVAs indicated a significant effect only at the peak time trial. Mutually orthogonal post-hoc comparisons of means obtained on this trial indicated that the means for saline treatments in conscious and anesthetized rats did not differ ($F=0.01$); the means of these two treatments combined differed significantly from the mean of DALA treatment in conscious rats ($F=4.31$); and that the means of the three preceding treatments combined did not differ from the mean of the DALA treatment in anesthestized rats ($F=0.00$). Thus, these analyses imply that hypotension occurs in the period prior to the 0.25-min time trial and to a greater extent in conscious compared to anesthetized rats.

The data for heart rate are presented in the bottom panel of Fig. 1. In general, DALA induced a larger peak decrease in heart rate in conscious compared to anesthetized rats, but this difference was not maintained during the following time points where bradycardia was still observed. Mean baseline heart rates obtained immediately prior to the test trials were: conscious-saline=518; conscious-DALA=515; pentobarbital-saline=419; and pentobarbital-DALA=430, BPM. Clearly, heart rates were lower in the anesthetized condition and this was confirmed by an ANOVA on baseline heart rates that indicated a significant effect of anesthesia, $F(1,8)=18.45$, but not of drug, $F(1,8)=0.63$. A mixed design ANOVA on percentage change measures obtained following administration of the drugs indicated a significant effect of drug, $F(1,8)=61.75$; a significant effect of time, $F(6,48)=36.57$; a significant drug × time interaction, $F(6,48)=17.30$; and a significant anesthesia × drug × time interaction, $F(6,48)=8.33$. One way ANOVAs then performed on each trial revealed significant differences at time points peak, 0.25-, 1-, 2-, 3-, and 4-min (Fs ranged from 6.42 to 34.52). Scheffe post-hoc comparisons of means revealed the following patterns of outcomes: At the peak time point and the 0.25-min time point, the two saline treatment means did not differ (Fs of 0.19 and 0.02); the saline treatment means combined differed significantly from the conscious DALA treatment mean (Fs of 18.18 and 6.20); and the latter three treatment means combined did not differ from the pentobarbital DALA treatment means (Fs of 0.09 and 0.19). On trials 1-, 2-, 3-, and 4-min the following pattern of outcomes were obtained: The two saline treatments did not differ (Fs ranged from 0.01 to 0.31); the two DALA treatment means did not differ (Fs ranged from 0.06 to 0.02); and the saline treatment means combined differed significantly from the two DALA treatment means combined (Fs ranged from 10.58 to 34.16). Thus, DALA did produce a larger bradycar-

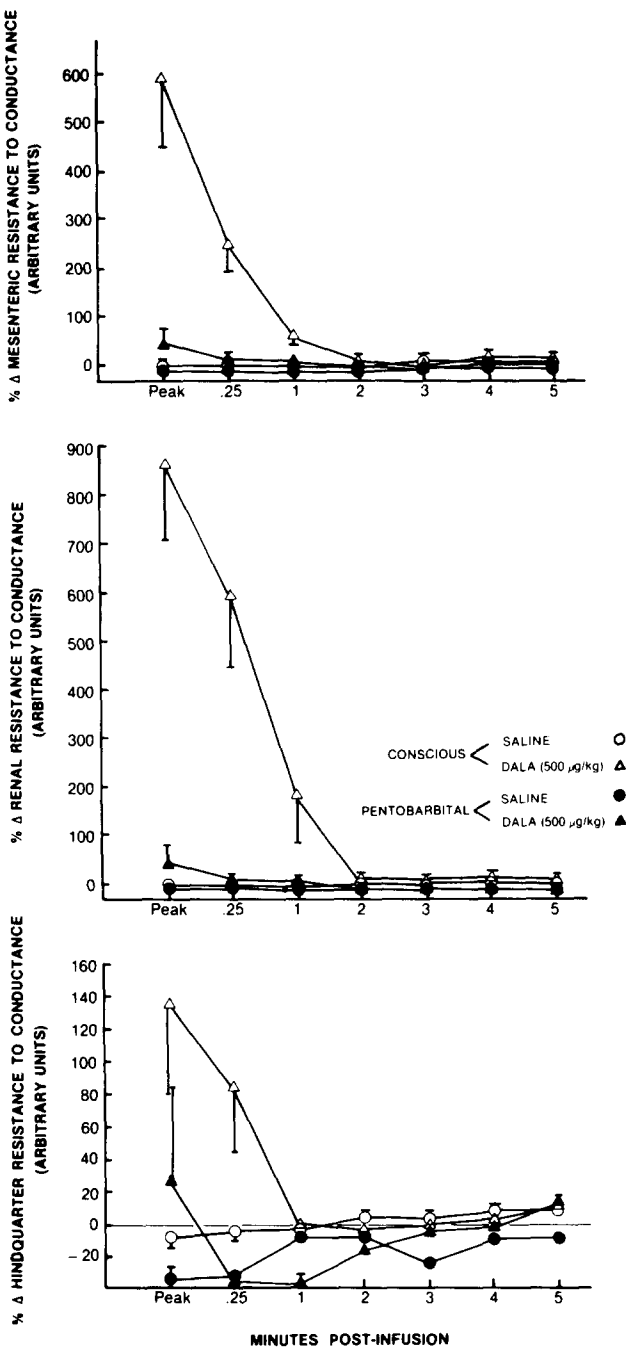

FIG. 3. Mean percent change in resistance to conductance measures (MAP/kHz of Doppler shift in arbitrary units) in the mesenteric (top panel), renal (middle panel), and hindquarter (bottom panel) vascular beds as a function of either saline or DALA (500 μg/kg) administration in conscious and pentobarbital-anesthetized rats.

dia in conscious compared to anesthetized rats immediately following drug administration, but these differences were not sustained during the periods when heart rate still remained significantly reduced compared to saline treatments.

Figure 2 presents mean percent change from baseline measures of blood flow using the superior mesenteric artery, renal artery, and the abdominal aorta as indices of blood flows in the mesenteric bed, the renal bed, and the hindquarter. Infusion of DALA resulted in immediate large decreases in flows of all these vascular beds of conscious rats, but generally smaller reductions in flow of pentobarbital-anesthetized rats (note the reduction in flow in the hindquarter is relative to saline values). In the mesenteric and renal beds, flows then returned to baseline values. However, there was a subsequent large increase in flow observed between 0.25–1 min in the hindquarter bed in every rat tested. Individual mixed design ANOVAs were performed on percentage change measures from each vascular bed and confirmed the impressions noted above.

In the mesenteric bed, a mixed design ANOVA indicated a significant effect of anesthesia, $F(1,10)=9.22$; a significant effect of drug, $F(1,10)=10.55$; a significant effect of time, $F(6,60)=39.79$; a significant anesthesia × time interaction, $F(6,60)=5.69$; a significant drug × time interaction, $F(6,60)=47.01$; and a significant anesthesia × drug × time interaction, $F(6,60)=6.14$. One way ANOVAs were significant on trials peak, 0.25-, and 1-minute, and post-hoc Scheffe analyses of means revealed the following pattern of outcomes: On these trials the means of the two saline treatments did not differ (Fs ranged from 0.05 to 0.22); the two saline means combined differed significantly from the mean of the conscious DALA treatment (Fs ranged from 5.03 to 26.23); and the latter three treatment means combined did not significantly differ from the mean of the pentobarbital DALA treatment (Fs ranged from 0.00 to 0.40). These results imply that DALA decreased flow to a greater extent in conscious compared to anesthetized rats.

In the renal bed, a mixed design ANOVA indicated a significant effect of drug, $F(1,10)=7.63$; a significant effect of time, $F(6,60)=34.72$; a significant anesthesia × time interaction, $F(6,60)=14.92$; a significant drug × time interaction, $F(6,60)=31.90$; and a significant anesthesia × drug × time interaction, $F(6,60)=10.17$. One way ANOVAs were significant on trials peak, 0.25-, and 1-min, and post-hoc Scheffe analyses revealed the following pattern of outcomes: On trials peak and 0.25-min, the two saline treatment means did not differ (Fs of 0.01–0.03); the two saline treatments means combined differed significantly from the conscious DALA mean (Fs of 26.57–40.27); and the latter three treatment means combined did not differ from the pentobarbital DALA treatment mean (Fs of 0.18–0.59). Thus, DALA exerted a stronger reduction in renal flow in conscious compared to anesthetized rats. On trial 1-min, the two saline treatment means did not differ ($F=0.00$); these two means combined did not differ from the pentobarbital DALA mean ($F=0.00$); and the latter three treatment means differed significantly from the conscious DALA mean ($F=3.32$).

In the hindquarter bed, a mixed design ANOVA indicated a significant effect of anesthesia, $F(1,10)=35.00$; a significant anesthesia × time interaction, $F(6,60)=3.30$; and a significant drug × time interaction, $F(6,60)=8.87$. One way ANOVAs were significant at times peak, 0.25-, and 1-min, and post-hoc Scheffe analyses revealed the following pattern of outcomes: On trials peak and 0.25-min, the means of the conscious saline treatment and the pentobarbital DALA treatment did not differ (Fs of 0.01–0.17); the means of the conscious DALA treatment and the pentobarbital saline treatment differed significantly (Fs of 9.50–3.95); and the combined means of conscious saline and pentobarbital DALA did not differ from the combined means of the conscious DALA and pentobarbital saline (Fs of 0.03–0.00). On trial 1-min, the two saline means did not differ (F of 0.26); the two DALA means did not differ ($F=0.71$); and the two saline treatment means combined differed significantly from the two DALA treatment means combined ($F=3.08$). These analyses indicate that at the peak and 0.25-min trials, DALA induces a drop in flow in both conscious and anesthetized rats relative to saline controls, but the baselines from which they move differ. In contrast, at the 1-min time point there is a significant increase in flow in both conscious and anesthetized rats relative to controls.

Analyses were then carried out on resistance to conductance measurements which were calculated as pressure/flow (kHz of doppler shift) with the limitation that the maximal arbitrary change in resistance to conductance was 1000% from baseline measures. Figure 3 presents the results of these analyses. In general, DALA resulted in an increase in resistance to conductance in all vascular beds, although the largest changes occurred in the mesenteric and renal beds. Moreover, pentobarbital drastically reduced the effects of DALA on these measures.

ANOVAs performed on percent change measures (arbitrary units) confirmed these views. In the mesenteric bed, there was a significant effect of anesthesia, $F(1,10)=12.02$; a significant effect of drug, $F(1,10)=13.04$; a significant anesthesia × drug interaction, $F(1,10)=9.78$; a significant effect of time, $F(6,60)=11.72$; a significant anesthesia × time interaction, $F(6,60)=9.23$; a significant drug × time interaction, $F(6,60)=12.95$; and a significant anesthesia × drug × time interaction, $F(6,60)=9.37$. Individual one-way ANOVAs indicated significant between-groups differences at the peak, 0.25-, and 1-min trials, and Scheffe post-hoc comparisons of means indicated the following pattern of outcomes: On all these trials, there were no differences between the means of the saline treatments or the combination of the saline treatments versus the pentobarbital DALA treatment (Fs ranged from 0.00 to 0.06); there were significant differences between the combination of the latter three means and the mean of the conscious DALA treatment (Fs ranged from 5.66 to 18.76). Thus, resistance to conductance was only significantly enhanced in the conscious rats treated with DALA at the peak, 0.25-, and 1-min trials.

A similar pattern of outcomes was obtained in the renal bed. The mixed design ANOVA indicated significant effects of anesthesia, $F(1,10)=16.41$; a significant effect of drug, $F(1,10)=14.92$; a significant anesthesia × drug interaction, 13.60; a significant effect of time, $F(6,60)=14.86$; a significant anesthesia × time interaction, $F(6,60)=13.84$; a significant drug × time interaction, $F(6,60)=15.53$; and a significant anesthesia × drug × time interaction, $F(6,60)=13.62$. Individual one-way ANOVAs indicated significant differences at the peak and 0.25-min trials, and Scheffe post-hoc comparisons of means indicated the following pattern of outcomes: On both these trials, the means of the saline treatments did not differ and the combination of these means failed to differ from the mean of the pentobarbital DALA treatment (Fs ranged from 0.00 to 0.04); the latter three means combined differed significantly from the mean of the conscious DALA treatment (Fs of 25.31 and 12.13). Thus, resistance to conductance was significantly increased only in

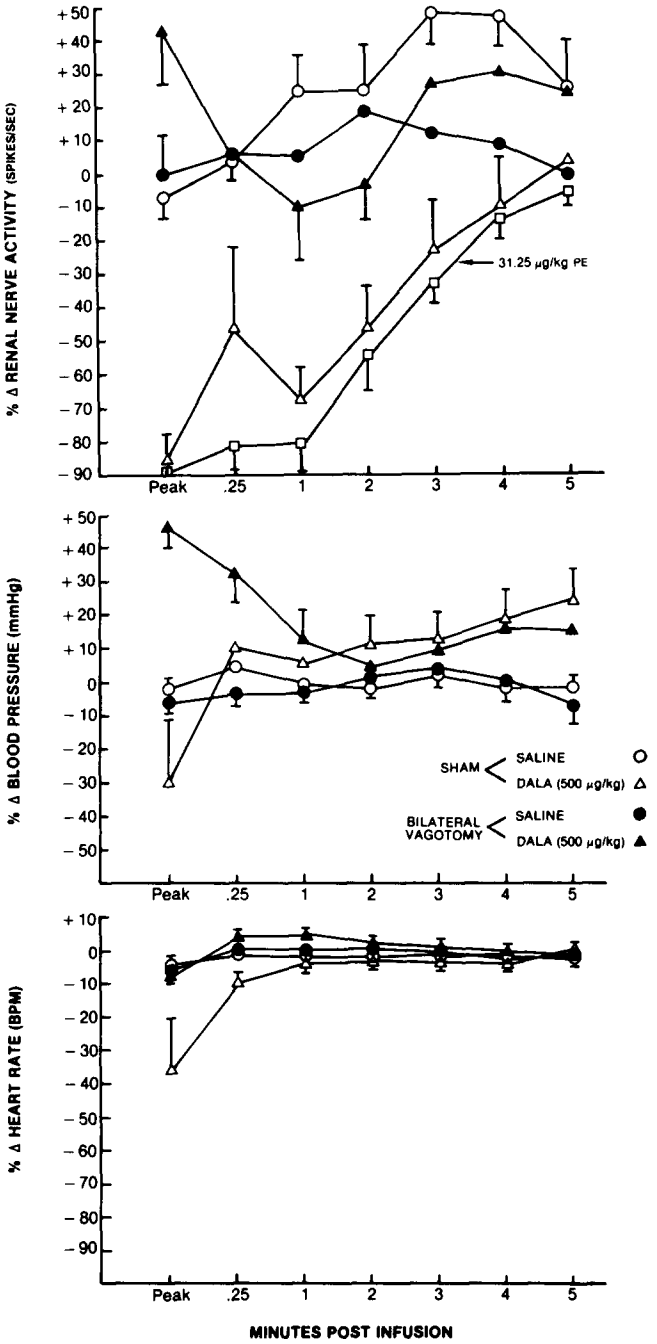

FIG. 4. Mean percent change in multiunit renal nerve activity (top panel), mean percent change in arterial blood pressure (middle panel), and mean percent change in heart rate (bottom panel) following either saline or DALA (500 μg/kg) administration in sham-operated and bilateral vagotomized rats under pentobarbital anesthesia. Comparison functions for 31.25 mg/kg of phenylephrine are provided in the top panel.

the conscious DALA treatment at the peak and 0.25-min trials.

In the hindquarter bed, the mixed design ANOVA indicated a significant effect of anesthesia, $F(1,10)=14.31$; a significant effect of drug, $F(1,10)=7.79$; and a significant drug × time interaction, $F(6,60)=4.41$. Individual one-way ANOVAs indicated significant differences at trials peak and 0.25-min, and Scheffe post-hoc comparisons of means indicated the following pattern of outcomes: On both of these trials, the two saline means did not differ and the combination of these means failed to differ from the mean of the pentobarbital DALA treatment (Fs ranged from 0.00 to 0.36); the latter three means combined differed significantly from the mean of the conscious DALA treatment (Fs of 3.21 and 4.70). Thus, resistance to conductance was significantly increased only in the conscious DALA treatment at the peak and 0.25-min trials.

Figure 4 presents data obtained from recordings of renal sympathetic nerve activity, blood pressure and heart rate following administration of either saline or DALA in sham-vagotomized or bilaterally vagotomized rats.

In sham-operated rats, renal sympathetic nerve activity decreased dramatically over the 5-min observation period and was accompanied by short-lived decreases in blood pressure and heart rate, similar to those observed in Experiment 1. In contrast, bilaterally vagotomized rats showed a brief increase in renal sympathetic nerve activity followed by a return to control levels with concomitant increases in arterial blood pressure and no change in heart rate. It is possible that the initial increase in renal nerve activity was rapidly inhibited by the pressure-induced activation of sinoaortic baroreceptors.

A mixed design ANOVA on percent change measures of renal nerve activity indicated significant effects of the operation, $F(1,9)=7.81$; a significant effect of drug, $F(1,9)=7.29$; a significant effect of time, $F(6,54)=6.93$; a significant operation × drug interaction, $F(1,9)=12.11$; a significant operation × time interaction, $F(1,9)=5.52$; and a significant drug × time interaction, $F(6,54)=3.42$. Individual one-way ANOVAs indicated significant effects at the peak, 1-, 2-, and 3-min trials, and Scheffe post-hoc comparisons of means indicated the following pattern of outcomes: At the peak measurement, activity produced by the two saline means did not differ ($F=0.07$); the two DALA means differed ($F=23.77$); and the combination of the two saline means failed to differ from the combination of the two DALA means ($F=0.98$). At the 1- and 2-min trials, the two saline means did not differ (Fs of 0.49 and 0.05); these two means combined did not differ from the vagotomized DALA mean ($Fs=1.02$ and 1.07); and the combination of these three means differed from the sham-operated DALA means ($Fs=10.51$ and 6.43). At the 3-min trial, the saline and DALA means for vagotomized rats did not differ ($F=0.12$); the saline and DALA means for the sham-operated rats differed significantly ($F=5.56$); and the combination of the vagotomized rats means compared to the sham-operated rats means did not differ ($F=0.06$). Thus, it is reasonable to conclude that DALA inhibits renal nerve activity in sham-operated rats, whereas it increases renal nerve activity in bilaterally vagotomized rats. However, the magnitude of the increase in renal nerve activity of vagotomized rats is probably attenuated by the increase in arterial blood pressure which engaged the high pressure baroreflex arc to diminish sympathetic nerve activity.

Mean baseline arterial blood pressures were sham-saline=103; sham-DALA=89; vagotomized-saline=112; and vagotomized-DALA=100, mmHg. An ANOVA indicated a significant difference with respect to drug, $(F1,9)=5.25$, indicating that baseline blood pressures were higher prior to the saline trials compared to the DALA trials. A mixed

design ANOVA on arterial blood pressures indicated a significant effect of drug, $F(1,9)=9.81$; a significant operation × time interaction, $F(6,54)=8.46$; and a significant operation × time × drug interaction, $F(6,54)=10.07$. Individual one-way ANOVAs indicated significant differences on the peak, 0.25-, and 5-min trials, and Scheffe post-hoc analyses indicated the following pattern of outcomes: At the peak trial, the two saline treatment means did not differ ($F=0.02$); the two DALA treatment means differed significantly ($F=8.15$); and the combination of the saline means failed to differ from the combination of the DALA means ($F=0.50$). At the 0.25-min trial, the two saline means did not differ ($F=0.27$); these two means combined did not differ from the sham-operated DALA mean ($F=0.76$); and the combination of these three means differed significantly from the vagotomized DALA mean ($F=5.32$). At the 5-min trial, the two saline means did not differ ($F=0.13$); the two DALA means did not differ ($F=0.35$); and the combination of the two saline means differed from the combination of the two DALA means ($F=4.79$). These analyses indicated that the initial peak hypotensive action of DALA observed in sham-operated rats is replaced by a peak hypertensive action in bilaterally vagotomized rats. Although renal nerve activity was elevated in bilaterally vagotomized rats at this time, administration of DALA also increased arterial blood pressure in rats pretreated with 20 mg/kg of hexamethonium, thereby indicating a possible peripheral vasoconstrictor action of DALA and/or an ability of DALA to non-neurogenically activate post-ganglionic sympathetic nerve fibers.

Mean baseline heart rates were sham-saline=382; sham-DALA=408; vagotomized-saline=461; and vagotomized-DALA=450, BPM. An ANOVA indicated significant baseline differences with respect to operation, $F(1,9)=6.56$. Thus, bilaterally vagotomized rats had significantly higher baseline heart rates than sham-operated rats. An ANOVA on heart rates indicated a significant operation × drug interaction, $F(1,9)=9.62$; a significant effect of time, $F(6,54)=8.15$; a significant operation × time interaction, $F(6,54)=2.79$; a significant drug × time interaction, $F(6,54)=4.35$; and a significant operation × drug × time interaction, $F(6,54)=2.52$. Individual one-way ANOVAs indicated significant differences on the peak and 0.25-min trials, and Scheffe post-hoc analyses indicated the following pattern of outcomes on these trials: On both of these trials, the saline means did not differ (Fs of 0.00 and 0.02) and the combination of these two means did not differ from the vagotomized-DALA mean (Fs of 0.03 and 1.41); the combination of the latter three means differed significantly from the sham-operated DALA mean (Fs of 3.64 and 11.72). Thus, bilateral vagotomy completely eliminated the normal bradycardic action of DALA.

GENERAL DISCUSSION

Several studies and investigators have suggested that systems involved in cardiopulmonary function are linked to systems controlling the perception of pain [1–3, 16, 17, 23–26, 32, 35, 36]. DALA is a compound that induces both autonomic changes and antinociception by peripheral activation of vagal afferents in the cardiopulmonary region [23, 33, 34], thereby providing a useful experimental stimulus for the analysis of cardiopulmonary-somatosensory interactions. The purpose of the present studies was to both quantify and delineate antinociceptive, cardiovascular, and autonomic nervous system actions of DALA in both conscious and anesthetized rats. The anesthetized rat preparation will likely be required to identify CNS substrates responsible for cardiopulmonary-somatosensory interactions.

Experiment 1 demonstrated that DALA induces antinociception in pentobarbital-anesthetized rats (see also [23,25]). Within the power considerations of the present design, DALA only induced significantly greater antinociception in pentobarbital-anesthetized rats compared to conscious rats 5-min after the drug infusion. TFI values of pentobarbital-anesthetized rats obtained at earlier test trials did not significantly differ from those manifested by conscious rats, although they tended to be consistently smaller. This could be interpreted as supporting the view that descending systems of pain inhibition are tonically active in conscious rats, and that barbiturate anesthesia releases these systems from tonic inhibition [29,30]. Under some circumstances, therefore, pentobarbital-anesthetized rats may be more responsive to noxious input than their conscious counterparts, and should be considered in the interpretation of results. For example, treatments that block the antinociceptive action of either a drug or electrical stimulation of CNS sites might be easier to demonstrate in the pentobarbital-anesthetized preparation. It should also be noted that the artificial ceiling of 10 sec, imposed to eliminate tissue damage, may have precluded detecting differences at the 0.25- and 1-min time trials. In general, however, the pentobarbital-anesthetized rat preparation appears to provide a useful model for the study of DALA-induced activation of CNS substrates of pain inhibition.

IV administration of DALA also results in an initial, short-duration hypotension (up to 15 seconds) followed by a return to baseline values. At this point, a mild hypertension may sometimes be observed, particularly in pentobarbital-anesthetized rats. A similar pattern of results and time course was obtained with injection of DALA into the right lateral ventricle followed by penetration of DALA into the systemic circulation [4]. The responses of pentobarbital-anesthetized rats were markedly blunted relative to conscious rats in all cases. The hypotension itself may primarily reflect a withdrawal of sympathetic tone to the vasculature, as indexed by a large (90%) peak decrease in renal sympathetic nerve activity. The large reflex bradycardic response induced by DALA probably contributes relatively little to the hypotension [33]. However, the initial hypotensive response clearly represents the summation of both depressor and pressor actions of DALA, and indeed, probably underestimates the pure depressor action of DALA. Specifically, three distinct blood pressure changes appear to provide a net outcome of hypotension. First, DALA activates vagal afferents to provide withdrawal of sympathetic tone, and this presumably is responsible for a depressor action that ultimately masks pressor actions of the drug. Second, DALA induces a brief increase in both blood pressure and renal sympathetic nerve activity of rats with bilateral vagotomies suggesting that DALA may act either on peripheral receptors other than vagal afferents (for example, sympathetic afferents to engage a sympatho-sympatho excitatory reflex) or indirectly to engage post-ganglionic sympathetic efferents. In a previous study [23], a similar hypertension was observed in rats with bilateral vagotomies, but was not analyzed because there were no saline controls. Third, DALA evoked pressor responses following administration of hexamethonium suggesting the possibility of a direct vasoconstrictor action of DALA as well. Nonetheless, the net result of all these effects is a brief hypotension in the intact

rat attesting to the strength of the vagal afferent input.

Heart rate was decreased by DALA and this effect is due to vagal afferent activation of vagal cardiomotor efferents [23,33]. Willette *et al.* [33] argued that the profound bradycardia induced by DALA does not contribute to the observed hypotension. As true for the arterial blood pressure, heart rate responses are dramatically reduced in the pentobarbital-anesthetized rat compared to the conscious rat.

The present studies also showed that arterial blood flows were dramatically reduced in the mesenteric, renal, and hindquarter vascular beds, and that the responses in conscious rats were significantly larger than in pentobarbital-anesthetized rats. Flow changes were more complex in the hindquarter bed. In conscious rats, DALA induced an immediate decrease in hindquarter flow followed by a significant increase in flow between 0.25 and 1 min. We observed this increase in every rat and the peak of this increase with the present parameters occurs between 30 and 45 sec after DALA infusion. A similar pattern of results occurs in pentobarbital-anesthetized rats, except that the functions are shifted with saline producing increases in flow. It is possible that this flow increase is artifactual, but similar changes were not simultaneously observed in the other vascular beds with exactly the same stimulus. We have no ready account for this shift except to note that the pattern, i.e., decreased flow followed by increased flow, was consistent across the anesthesia treatment. Moreover, the fact that antinociception occurred in pentobarbital-anesthetized rats with virtually no change or an increase in hindquarter flow indicates that inadequate flow to the cord does not contribute to the elevations in latencies to tail-flick. This is also consistent with our observation that bilateral lesions of the DLF block DALA-induced inhibition of the tail-flick reflex [25], where rapid tail-flick responses occur in the presence of reduced flows.

There were large increases in resistance to conductance measures in all vascular beds of conscious rats, but these responses were dramatically blunted or not significant in pentobarbital-anesthetized rats. The latter outcomes are again consistent with the view that elevated tail-flick latencies following DALA administration are not a consequence of reduced flows to the beds. However, the resistance to conductance measures are difficult to interpret in the present situation. It may well be the case that the dramatic reduction in cardiac output and arterial flows brought about by the large reflex bradycardia is primarily responsible for the resistance to conductance increases. However, these changes may also reflect resistance changes in the vascular beds due to direct actions of DALA or secondary hormonal influences. These views are compatible with our observations that DALA exerts pressor actions in the absence of vagal afferents. If there is a resistance change, however, it occurs in the face of massive withdrawal of sympathetic tone (as indexed by renal sympathetic nerve activity). Recall that the magnitude of this withdrawal was similar to that produced by a bolus infusion of 31.25 μg/kg of phenylephrine which raised arterial blood pressure an average of 72 mmHg at the peak time point and decreased heart rate by 33%. These outcomes clearly attest to the capacity of vagal afferents to alter sympathetic outflow.

It is our opinion [24,25] that these changes in nociception and cardiovascular function should be considered as reflex changes, both induced by increased activity of vagal afferents. In the present analyses, however, the cardiovascular but not the antinociceptive effects of DALA were markedly blunted in the pentobarbital-anesthetized rat compared to the conscious rat. This suggests that the initial antinociceptive response induced by DALA is relatively unaffected by the transient hypotension seen as a cardiovascular response and possibly operating secondarily to unload sinoaortic and/or cardiopulmonary baroreceptors to reduce baroreceptor activity and hence, antinociception.

In conclusion, these studies will aid future research of CNS substrates of visceral afferent-somatosensory interactions by both characterizing and establishing the viability of the pentobarbital-anesthetized rat preparation. Primary vagal afferents terminate in and around the region of the nucleus tractus solitarius (NTS:) [15]. Lewis, Baldrighi, Watson, and Akil (Lewis, J. W., G. Baldrighi, S. J. Watson and H. Akil. Electrical stimulation of the nucleus tractus solitarius (NTS) causes opioid mediated analgesia in the rat. *Soc Neurosci Abstr* **11:** 637, 1985) have reported that electrical stimulation of the NTS results in an opioid-mediated antinociception in the rat. Randich and Maixner [25] demonstrate that descending fibers controlling inhibition of the tail-flick reflex following administration of DALA are in the dorsolateral aspect of the lateral funiculus (DLF), although apparently descending control of sympathetic outflow produced by DALA administration is in a different portion of the cord [25]. Therefore, if descending inhibition is not mediated by a direct NTS-spinal cord projection, then other likely NTS-medullary-spinal substrates include the C1, A2, A5, B1, B2, and B3 relay areas. For example, Yen and Blum [36] discovered neurons that selectively respond to both high pressure baroreceptor input and noxious pinch in the medullary raphe nuclei of cat. These cells then send fibers down the cord to presumably modulate both cardiovascular function and somatosensory input. The present preparation will permit analyses of these areas using techniques such as reversible anesthetic blocks and microinjection of agonists and antagonists.

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